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NANOPARTICLE ENCAPSULATION OF BACTERIAL ANTIGEN FOR VACCINATION PURPOSES

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Master thesis

Supervisors: Dr. Carlos Gamazo, PharmDr. Petr Jílek, CSc.

Pamplona 2016

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NANOČÁSTICOVÁ ENKAPSULACE BAKTERIÁLNÍHO ANTIGENU PRO POUŽITÍ VE VAKCÍNÁCH

Alžběta Nemeškalová

Diplomová práce

Vedoucí diplomové práce: Dr. Carlos Gamazo, PharmDr. Petr Jílek, CSc.

Pamplona 2016

”I confirm that this Master’s thesis is my own work and I have properly documented all sources and material used. This thesis was not previously submitted for any academic degree and has not been published.”

„Prohlašuji, že jsem diplomovou práci vypracovala samostatně za použití uvedených pramenů a literatury. Dále prohlašuji, že tato práce nebyla použita k získání jiného nebo stejného titulu.“

Hradec Králové
April 25, 2016

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I would like to express my gratitude to my supervisor, Dr. Carlos Gamazo, for his guidance, kindness and patience, and most of all for his ability to arouse enthusiasm for research in the most unlikely person - me.

Abstract

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Shigellosis, an infectious diarrheal disease caused by *Shigella spp.*, is currently a major health concern in developing countries and there is still no vaccine available. Non-living vaccines seem to be the safest option: our approach is therefore based on using Outer membrane vesicles (OMVs) as a subunit vaccine. However, mucosally administered OMVs are not capable of inducing as appropriate protection and a suitable adjuvant must be added to modify the immune response. Polymeric nanoparticles (NP) were studied in this work as mucosal adjuvats.

The aim of this project was to prepare particles based on a foodborne protein (Protein P, under patent). Apart from simple proteic particles, two different ligands (Compound A, Compound B) were used to modify the particle surface, which resulted in four different formulations (P, P-A, P-B, P-A-B). We loaded these NPs with OMVs and described their properties, such as size, polydispersity and Z-potential. Their capability to encapsulate and carry the bacterial antigens was also determined. Furthermore, a method of dissolving proteic NP was developed in order to reveal the total amount of encapsulated OMVs.

Our findings show that Protein P nanoparticles (size ranging from 222.9 ± 1.6 nm to 402.1 ± 0.7 nm) were able to encapsulate OMVs and carry it on their surface as well, no significant difference between each formulation was observed. This supports the idea of using the combination of OMVs and proteic nanoparticles as a vaccine. Further experiments are carried out at the University of Navarra, determining the expected immunogenic properties.

Abstrakt

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Název diplomové práce: Nanočásticová enkapsulace bakteriálního antigenu pro vakcinální účely
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Shigelóza je infekční průjmové onemocnění způsobené rodem *Shigella*, které v současné době představuje významný problém pro rozvojové země a proti němuž neexistuje možnost očkování. Subjednotkové vakcíny jsou považovány za nejbezpečnější možnost: náš přístup zahrnuje použití OMV (Outer membrane vesicles, vezikuly vnější membrány) jako antigenu v podjednotkové vakcíně. Nicméně při mukosálním podání nejsou OMV schopny vyvolat dostatečný protektivní účinek a proto je nutné přidat vhodný adjuvans za účelem zvýšení imunitní odpovědi. Tato práce se zabývá použitím polymerních nanočástic jako slizničních adjuvans.

Cílem práce bylo připravit částice z přírodního proteinu (Protein P), získávaného z běžně se vyskytujícího zdroje potravy. Kromě Proteinu P byly použity dvě další sloučeniny, u kterých se předpokládá navázání na povrch částice. Díky tomu bylo možné připravit čtyři různé typy nanočástic (P, P-A, P-B, P-A-B), do kterých se následně přidal bakteriální antigen - OMV, a pozorovaly se základní vlastnosti výsledného produktu (velikost, polydisperzita a Z-potenciál). Následně byla zkoumána schopnost částic nést antigen, a to hlavně na svém povrchu. Byla také vyvinuta metoda, kterou bylo možné rozpustit Protein P a odhalit tak celkové množství antigenu obsaženého v částicích.

Výsledky ukazují, že částice připravené z Proteinu P (s velikostí od 222.9 ± 1.6 nm do 402.1 ± 0.7 nm) byly schopné enkapsulace OMV a zároveň schopné nést antigen na svém povrchu. V tomto ohledu nebyly pozorovány rozdíly mezi jednotlivými typy nanočástic. Výsledky tedy podporují použití kombinace proteinových nanočasic a OMV ve vakcíně. Další experimenty na Navarské univerzitě budou zkoumat jejich imunologické vlastnosti.

Contents

List of Abbreviations

BSA bovine serum albumine

CT Cholera toxin

DiO dioctadecyloxacarbocyanine perchlorate

DMSO dimethylsulfoxide

GM-CSF granulocyte-macrophage colony-stimulating factor

GRAS generally recognized as safe

H_2Od dionized water

H_2Odd double dionized water

IFNγ interferon gamma

IL interleukin

ISCOM immunostimulating complex

KDO 2-keto-3-deoxyoctonate

LPS lipopolysaccharide

LT heat-labile *Escherichia coli* enterotoxin

MOPS 3-(N-morpholino)propanesulfonic acid

NP nanoparticle

ODN oligodinucleotide

OMV outer membrane vesicle

PAGE polyacryamide gel electrophoresis

PBS phosphate-buffer saline

PLGA poly(lactic-coglycolic acid)

PMN polymononuclear neutrophil leukocyte

PVDF polyvinylidene difluoride

RCF relative centrifugal force

RT room temperature

SDS sodium docecylsulphate

TBS tris-buffered saline

TLR toll-like receptors

TNFα tumor necrosis factor alpha

1. Overview

This work was carried out during my four month Erasmus stay in Pamplona and the experiments were conducted at the Department of Microbiology and Parasitology of the University of Navarra (UNAV), under the supervision of Carlos Gamazo. Though I personally conducted the experiments described in chapters 4.2 and further, I still received a great amount of help and advice from my supervisor and all other members of his team, especially Yadira Pastor and Ana Brotons. And even though I present this paper as a work of my own, I would like to put forward the idea of a teamwork by using the word "we" instead of "I".

The team of Carlos Gamazo is currently studying polymeric nanoparticles and their use as adjuvants in a vaccine against shigellosis and porcine *Escherichia coli* infection. In the past, they focused on nanoparticles prepared from a synthetic polymer (Gantrez® AN) with very good results [1]. A new polymer is under research, being a foodborne protein, which is considered safe, cheap and readily available. As the results of their work are susceptible of patent protection, the nature of this protein and some other compounds used in this work cannot be described. This is why the names Protein P, Compound A and Compound B are used.

My role in this team was to prepare various nanoparticles based on this protein, load them with *Shigella* antigens and determine how composition affect their properties. In the Theoretical part of this paper, I will introduce the reader to shigellosis and attempt to portray what difficulties there are to overcome when developing a *Shigella* vaccine. The Experimental part contains description of experiments and used methods. Following my supervisor's advice, I will try to focus on explaining why we conducted these experiments and will describe only basic principles, so that the reader could follow our thoughts. Detailed descriptions of methods, including list of used material and equipment, can be found in the Appendix. In the following Results section, I will present outcomes in a simple and unbiased manner, while in the Discussion I shall comment on the results and compare them to our expectations. Chapter Conclusions summarizes the whole work by pointing out important observations and suggestions for further research.

2. Theoretical part

Diarrhoeal diseases caused by bacterial, viral or eucaryotic pathogens are one of the major health issues, with 1.7 billion cases globally reported by WHO every year. It is also a second leading cause of death in children under five, with an estimated 760,000 deaths per year [2]. Most of these deaths occur in developing countries in Africa and South Asia, where poor sanitation and lack of clean drinking water contribute to the spread of disease. Preventive measures to reduce the risk of the disease therefore include improved hygiene, promotion of exclusive breastfeeding, improved water supply and also vaccination [3].

2.1 *Shigella* spp.

Together with *Vibrio cholerae*, *Clostridium difficile*, variety of *Escherichia coli* serotypes, *Salmonella* spp. and *Campylobacter* spp., *Shigella* spp. is one of the main bacterial agents of diarrhoea in many countries worldwide [4], causing a wide range of symptoms from mild watery diarrhoea to severe inflammatory dysentery. It is a Gram negative, non-motile and non-spore forming rod shaped bacteria from the *Enterobacteriaceae* family, naturally occuring only in humans. *Shigella* is estimated to cause around 700,000 deaths each year, the majority of them ($\sim 60\%$) being children less than five years of age in developing countries [5]. This high disease burden and growing antibiotic resistance [6, 7] lead the fact that the need for a vaccine against shigellosis is a high priority.

There are 4 *Shigella* species: *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*, classified on the basis of O antigen component of lipopolysaccharide (LPS) present in the outer membrane. The first three mentioned are physiologically similar and consist of various serotypes, while *S. sonnei* differs from the other species by certain biochemical reactions and consists of one serotype only [8]. *S. dysenteriae* type 1 produces Shiga toxin and, though very rare in industrial countries, causes epidemic dysentery in the developing world. *S. flexneri* is endemic in developing countries, where it is the most frequently isolated species, while *S. sonnei* is on the other hand the main causative agent of shigellosis in the developed world [9]. Shigellosis caused by *S. boydii* is uncommon.

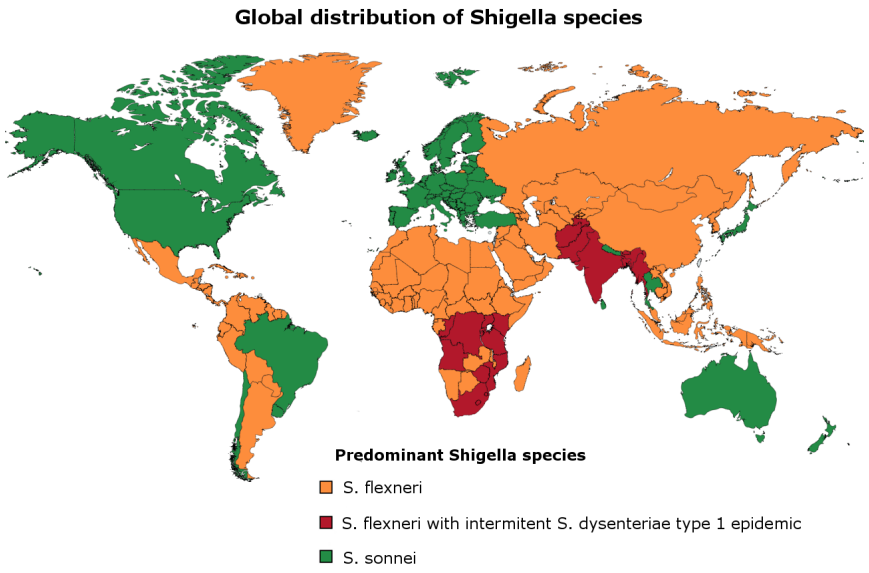


Figure 1: Global distribution of *Shigella* species. Map shows distribution of *S. flexneri*, *S. sonnei* and epidemics of *S. dysenteriae* type 1. Shigellosis caused by *S. boydii* (not displayed) is uncommon. Data source: Selendy, J. [10], created with Mapchart.

2.2 Shigellosis

Shigellosis, also called bacillary dysentery, can be developed 1–2 days after ingesting *Shigella*, with the fecal-oral route of transmission being the most common means of the disease transmission (other ways inlude ingestion of contamined water or food). Certain kinds of insect can also contribute to the spread by physically transporting contaminated material. The bacteria is considered highly infectious as a very little amount (10–100 organisms) [5] is sufficient to cause the disease.

After *Shigella* is ingested, it reaches the colon and crosses the epithelial barrier through the M cells, specialised cells which sample particles and transport them from gut lumen to immune cells. After exploiting the M cells as a way of translocation, *Shigella* induce cell death in macrophages, accompanied by the release of proinflammatory signalling. *Shigella* is then able to bind to the basolateral side of the epithelial cells and move into the cytoplasm of the enterocytes, causing the intercellular dissemination. The cytokines released by macrophages and epithelial cells during the bacterial invasion, IL-1β, IL-18 and IL-8 play part in inflammatory response and also lead to the recruitment of polymononuclear cells (PMN) which cross the epithelial barrier by disintegrating the tight junctions between enterocytes. This facilitates a massive entrance of bacteria and also exacerbates the infection. [11, 12]

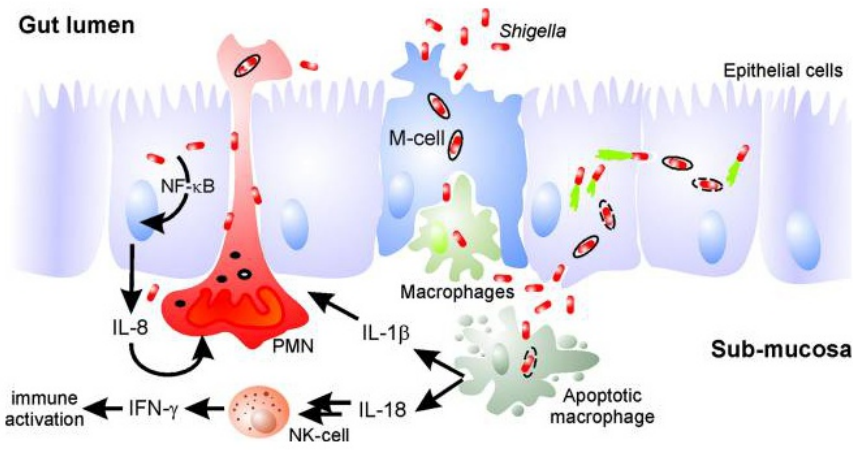


Figure 2: Pathogenesis of *Shigella* infection. *Shigella* crosses the epithelial barrier through M cells, attacks resident mactophages and induce apoptosis of macrophages accompanied by proinflammatory signaling. This further activates the innate immune response and attracts polymononuclear neutrophil leukocytes (PMN). The influx of PMN disintegrates the tight junctions between the cells which exacerbates the infection. Taken from Schroeder, G. N. [11]

Clinical signs are a result of colon inflammation, usually occur within 24–48 hours after the ingestion of bacteria and may range from mild abdominal discomfort to dysentery with typical afflictions such as abdominal cramps, blood and mucus in the stools and tenesmus. These are often accompanied by non-specific symptoms such as fever, anorexia and fatigue. The infection is usually self-limited and a healthy immunocompetent patient can recover within 7–10 days, although the infection can be life-threatening if the patient is immunocompromised or if no healthcare is available.

2.3 Vaccination

As mentioned above, improved hygiene and clean drinking water are crucial for preventing shigellosis, but considering a very low infectious dose and the fact that the disease is widespread in developing countries, these measures might be difficult to implement. This indicates a high priority need of vaccination, which could significantly reduce the spread of the disease.

Previous studies demonstrated that parenteral immunization with live or killed *Shigella* bacteria was insufficient to protect humans [13, 14]. Nowadays we can talk about two fundamental approaches: living attenuated and non-living vaccines, including dead bacteria and subunit vaccines. In the past decades only two have reached phase III of

clinical trials: oral administration of attenuated strains and parenteral administration of conjugates, which used *Shigella* O-polysaccharide covalently linked to a carrier protein. Unfortunately, major obstacles such as safety issues and lack of knowledge prevented licensing these candidates [15].

The mucosal route of administration of a vaccine against *Shigella* proves to be optimal in many respects. The reason for using a mucosal route of vaccination is mostly that most infections affect a mucosal surface and such vaccines are effective in inducing both mucosal and systemic immune response. There are further benefits of mucosal vaccination, including easier administration (or even the possibility of self-administration) and no need for needles or healthcare professionals. Oral vaccines are nowadays considered optimal for widespread use against enteric infections.

However, there are serious concerns about mucosal vaccines. Mucosa-administered antigens are generally less imunogenic and tend to induce tolerance. Moreover, other disadvantages include enzymatic degradation, lack of control of the delivered dose and higher amount of antigen required [16]. Indeed, only a few mucosal vaccines against enteric infections are currently approved for human use: oral vaccines against *Vibrio cholerae*, *Salmonella enterica* serovar *Typhi*, poliovirus and rotavirus, while vaccines against the two other most important causes of disease, enterotoxigenic *Escherichia coli* and *Shigella*, are still lacking. Full list of approved available mucosal vaccines can be found in Tab. 1.

2.3.1 Development of subunit vaccines

LPS, the major surface antigen and also the major virulence factor, is the main target for the innate immunity [18]. LPS is recognized by toll-like receptors (TLR) present on the surface of dendritic cells, which leads to production of proinflammatory factors, such as variety of interleukins, tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ). Furthermore, LPS activates antigen presenting cells, which express co-stimulatory molecules, CD80 and CD86. The presence of these molecules, together with TNFα and IFNγ leads to induction of adaptive immunity by activation of T-cells.

The presence of LPS seems to be essential in the development of a vaccine against *Shigella*. However, previous studies showed that immunization with LPS alone is ineffective. For this reason other additional antigens, for example outer membrane proteins, must also be considered [19].

Pathogen		Composition	Trade name (Company)
Vibrio cholerae	O*	Inactivated *V. cholerae* O1 (Inaba and Ogawa ser.)	Dukoral (SBL Vaccin AB)
Vibrio cholerae	O*	Killed whole cells of *V. cholerae* O1 and O139	Shanchol (Shantha Biotech.)
Rotavirus	O*	Live attenuated rotavirus type p1a (8), g1-g4	Rotateq (Sanofi-Pasteur)
Rotavirus	O*	Live attenuated rotavirus type rix 4414	Rotarix (GSK)
Salmonella enterica serovar *Typhi*	O*	Attenuated live strain of *S. typhi* Ty21a	Vivotif (Crucell)
Influenza	N*	Live attenuated influenza virus	Flumist (Medimmune)
Poliovirus	O*	Live attenuated virus Type 1 (LSc, 2ab) and Type 3 (Leon 12a, 1b)	Polio Sabin 1 & 3 (GSK)**

Table 1: List of commercially available vaccines administered by the mucosal routes. Other mucosal vaccine candidates for pathogens (*Bordetella pertussis, Enterotoxigenic Escherichia coli (ETEC), Vibrio cholerae, Shigella sonnei, Helicobacter pylori*, campylobacter, *Salmonella* Typhi and Paratyphi, *hemophilus influenzae* type B, and norovirus) have been recently tested in human trials.
*O=oral, N=nasal. ** The oral Polio Vaccine Sabin is not commercially available except in epidemic context. Taken from Nizard, M. [17]

2.3.2 Use of outer membrane vesicles

The use of outer membrane vesicles (OMVs) in vaccination belongs to the category of non-living subutit approaches and is currently very attractive. OMVs are small spherical structures (about 20–250 nm in diameter) released by Gram negative bacteria. They consist of proteins, such as porins and receptors, and lipids, such as LPS, phospholipids and lipoproteins. The OMVs of Gram-negative bacteria show to have various physiological functions, including formation of biofilms and transmitting virulence factors into host cells [20].

As OMVs are composed of many immunogenic molecules and can be recognized by immune system, their use as antigens in vaccines has become an attractive option. Nowadays, OMV-vaccines are successfully used against meningococcal group B disease and other vaccination candidates are currently studied: *Vibrio cholerae*, *Salmonella spp.* and *Shigella spp.* among many others [21]. An OMV-based vaccine containing *Shigella flexneri* 2a antigen has been successfully tested in mice, where one single dose of antigenic complex administrated by oral, ocular or nasal route induced high levels of protection against the infection [1].

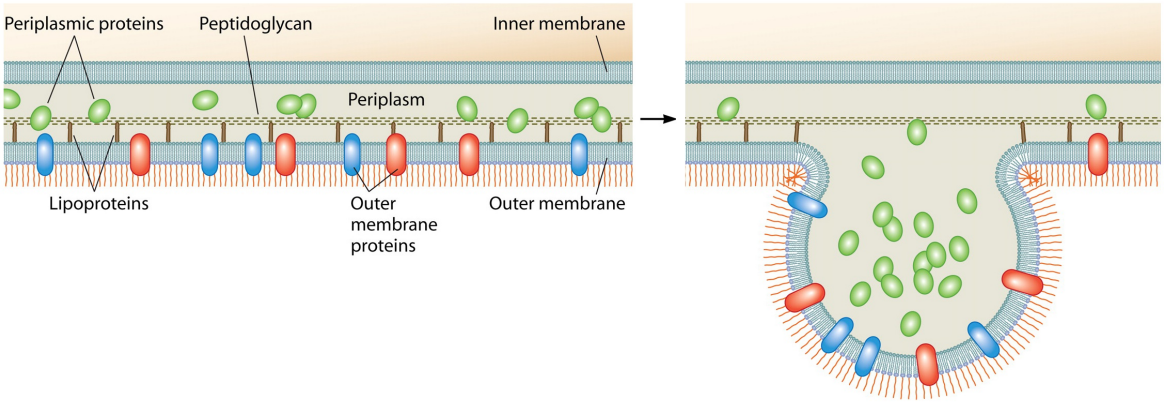


Figure 3: Secretion of outer membrane vesicles: bulging of Gram-negative bacterial envelope and releasing outer membrane vesicles (OMVs). Released OMVs contain periplasmic material and outer membrane proteins and lipids, including PAMPs and other virulence factors. Taken from Ellis, T. N. [22], modified.

2.4 Vaccination adjuvants

An important disadvantage of orally administered OMVs should be mentioned. When used orally as free antigens, they have problems reaching the gastrointestinal tract and inducing an appropriate immune response. Such poor induction of mucosal immunity therefore requires use of adjuvants systems, which can serve as vehicles or enhancers of immune response. An immunologic adjuvant can be defined as a compound which enhances and modulates the immunogenicity of the used antigen. There are several properties that a successful adjuvant should possess. An effective adjuvant should be non-toxic at the required dose, stimulate a strong humoral or cell mediated immunity and provide good immunological memory. At the same time, it should not be mutagenic, carcinogenic or teratogenic, and not induce autoimmunity and hypersensitivity. Finally, it should be stable under broad range of temperature, pH and storage periods [23].

Many diverse compounds have been used or tested as adjuvants in vaccines (see Tab. 2); however, not all of them are suitable for mucosal use. For instance, aluminium salts, currently the most widely used adjuvants in human and veterinary vaccines, [24] are effective when used through the parenteral route but ineffective when administered orally or nasally [25]. This is why a suitable mucosal adjuvant has to be chosen for the OMV-vaccine, though safe and effective mucosal adjuvants are still lacking.

There are two broad categories of currently studied adjuvants: (i) immunostimulatory adjuvants, including bacterial toxins and cytokines, which stimulate the immune response by interacting with specific receptors, (ii) delivery systems, such as micropar-

Name (licence year)	Adjuvant class	Components
Alum (1924)	Mineral salts	Aluminium phosphate, aluminium hydroxide
MF59 (1997)	Oil-in-water emulsion	Squalene, polysorbate 80, sorbitan trioleate
AS03 (2009)	Oil-in-water emulsion	Squalene, Tween 80, α-tocopherol
AS04 (2005)	Alum-adsorbed TLR4 agonist	Aluminium hydroxide, MPL
Virosomes (2000)	Liposomes	Lipids, hemagglutinin

Table 2: List of adjuvants approved for human use. Taken from Lee, S. [28]

ticles, nanoparticles, liposomes and other particulates, which enhance the immune response by antigen encapsulation, protection and release control [26]. Some compounds, though, can work by utilising both mechanisms simultaneously [27].

2.4.1 Immunostimulatory adjuvants

In the category of immunostimulants, perhaps the best-studied and also most potent mucosal adjuvants are the bacterial enterotoxins, particularly Cholera toxin (CT) and heat-labile *Escherichia coli* enterotoxin (LT), but they are considered too toxic for mucosal use in humans. In 2001 an enterotoxon-adjuvanted inactivated subunit nasal influenza vaccine (Nasalflu, Berna Biotech) was withdrawn from market after a considerable number of Bell's palsy was reported [29] and it is suggested there is a link between these enterotoxin-based adjuvants and inflammatory responses in the nasal tract [30]. This is why mutants or derivatives of CT and LT, either fully non toxic or with reduced toxicity, have been developed and studied. While their immunogenic effect was demonstrated on nasal administration, their oral adjuvanticity shows limitations [31].

Cytokines and chemokines, small proteins important in cell signalling, can also act as mucosal adjuvants. Generally, most adjuvants work by inducing production of inflammatory cytokines and more potent adjuvants will further stimulate this production in both quantity and variety. One approach to deal with overly toxic adjuvants is to copy the signals they induce by simply adding these signalling molecules. Although cytokines/chemokines show adjuvant properties by themselves, they also seem to have a synergic effect when used in combinations. Studies show that IL-1 in combination with Th1-inducing cytokines such as IL-12, IL-18, and granulocyte-macrophage colony-stimulating factor (GM-CSF) can induce as strong mucosal and systemic responses as

CT [32]. Apart from these, synthetic oligodinucleotides (ODNs) containing unmethylated CpG motifs represent another promising type of adjuvant. They have been found to enhance innate and adaptive mucosal immunity in animal models after nasal, oral or vaginal administration, however, CpG ODNs are currently still mainly considered for systemic use [16].

2.4.2 Nanoparticle delivery system

As described above, none of the previous categories of adjuvants show satisfying results. Therefore, attention has been recently shifted towards using nanoparticles (NPs) as delivery vehicles and a large scale of particles have been developed as alternatives to immunostimulatory adjuvants. Particulate delivery systems include various types of nanoparticles, microparticles, liposomes, immunostimulating complexes (ISCOMs) and other particles formed from biocompatible and biodegradable polymers. Such delivery systems are capable of protecting the antigen from degradation as well as increasing the antigen uptake by antigen presenting cells [33].

Generally NPs are solid particles ranging from 10 nm to 1000 nm in size and the vaccine antigen is either encapsulated within the particle or is present on the surface. By encapsulating the antigen, NPs provide protection and a means to transport antigens, which would otherwise rapidly degrade. Conjugation of antigen onto the particles surface mimics presenting the antigen to immune cells in a way it would be presented by pathogen itself. In addition, prolonged release is a desired feature as it can maximize exposure to the immune system. As mentioned above, NPs of various compositions have been prepared and studied. Examples are shown in Fig. 4, yet our studies are particularly focused on polymeric NPs.

Polymeric nanoparticles are solid particles with size ranging from $10-1000$ nm (nanoparticles) or $1-100$ μm (microparticles). A great variety of polymers are used for NP preparation: natural proteins (i.e. albumin) or natural polysaccharides (i.e. chitosan), as well as synthetic polymers. For instance, poly(lactic-coglycolic acid) (PLGA) is a biodegradable and biocompatible synthetic polyester used for many years as a controlled release drug delivery system in humans. The adjuvant effect of PLGA-entrapped antigen was demonstrated on antigens derived from various pathogens, including *Bacillus anthracis* [35] and hepatitis B virus [36].

The polymer which was previously studied at Navarra university is a poly(methyl vinyl ether/maleic anhydride) copolymer, available under trade name Gantrez®AN (Ashland Inc., KY, US). This copolymer is listed as "generally recognized as safe" GRAS product and is used in pharmaceutical and cosmetic industry as bioadhesive

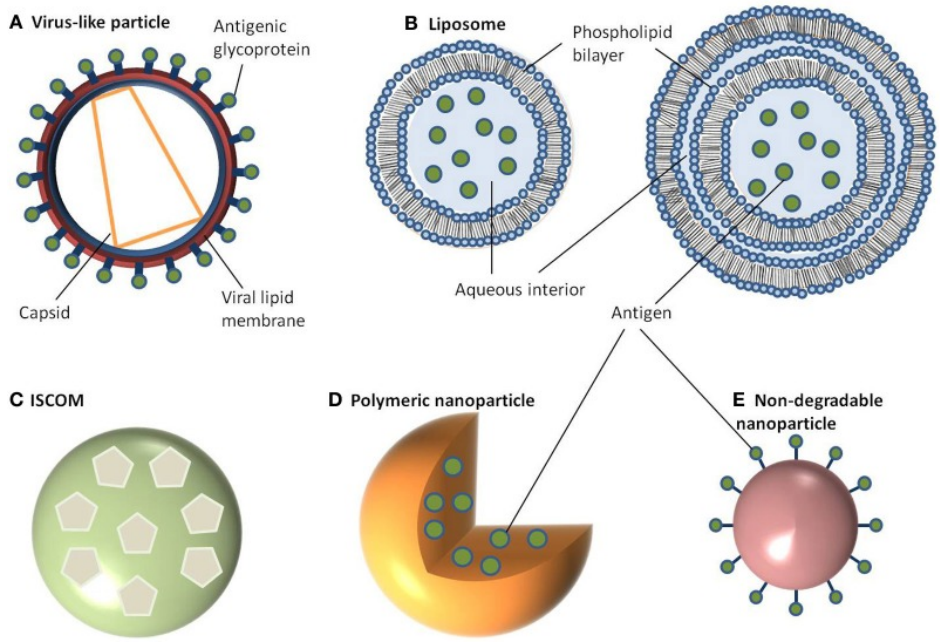


Figure 4: Schematic representation of different particulate delivery systems. (A) Virus-like particle, (B) Liposome, (C) Immunostimulating complex (ISCOM), (D) Polymeric nanoparticle, (E) Non-degradable nanoparticle. Taken from Gregory, A. E. [34]

material, emulsion stabilizer, film-forming agent and viscosity-increasing agent [37]. Previous studies demonstrated that encapsulation of OMVs from *S. flexneri* 2a into Gantrez®AN NPs increased protection in mice [1]. However, the mucoadhesive effect of these NPs appear to be limiting, as an important part of administered remains trapped in the protective mucus layer. This decreases the access of loaded antigen to the epithelial surface.

This is why new nanoparticle formulations are currently studied by our group. Instead of a synthetic polymer, a natural foodborne protein was chosen. The decision to use this protein was based on the safety issues, as it is originated in a common food source, it belongs to substances generally recognized as safe (GRAS). Besides, it is cheap and widely available. In addition, several ligands are added to the formulation. These compounds are believed to bind onto the proteic surface and their ability to modify bioadhesive and adjuvant properties is currently being studied. However, results of those studies have not yet been published and therefore the nature of these compounds cannot be described.

3. Aims of this work

The overall objective of this project is to develop a safe, effective and low-cost vaccine against shigellosis, using outer membrane vesicles (OMVs) encapsulated into nanoparticles. The method of preparing these nanoparticles was developed previously, however, it was not specifically designed for our antigen. Our goal is to confirm that these nanoparticles possess an adjuvant effect and are suitable for OMV delivery. Thus, their capability to encapsulate and carry OMVs must be determined.

Four nanoparticle types were produced in this work, two of them (types P and PAB) had been previously studied, the other two (types PA and PB) are new. Before their adjuvanticity studies, it is necessary to confirm that all these NPs are able to carry the *Shigella* antigen.

The aims of this work can be summarized in these main points:

- To prepare empty nanoparticles using foodborne Protein P and add Compound A and Compound B to their surface to create four different NP types (named P, PA, PB and PAB)
- To prepare same types of nanoparticles loaded with OMVs
- To characterize and compare these nanoparticles in terms of size, Z-potential and amount of encapsulated antigen, and observe the effect of Compound A and Compound B on those properties

4. Experimental part

OMVs, the bacterial antigen used for the experiments (section 4.1), were obtained from the Department of Microbiology and Parasitology, University of Navarra. As a first step, basic properties of OMVs were described (see 4.2), as they might affect further experiments or influence the properties of the final product. The next step was the preparation of our nanoparticles (section 4.3), which took place at the Department of Pharmaceutical Technology, University of Navarra. Each nanoparticle type was prepared in its OMV loaded and empty form, the loaded form being the examined material and the empty form as a reference and control. Basic characteristics (size and Z-potential) were examined immediately after preparation. The next steps were focused mainly on the encapsulated OMVs: First we located the OMVs on the nanoparticles surface (section 4.4), as their presence may significantly influence the final effect on immune cells. Next, the total amount of antigen was determined. A method of dissolving the nanoparticles had to be developed (section 4.5) in order to reveal the total encapsulated antigen, which was subsequently examined (section 4.6).

4.1 Extraction of the Outer membrane vesicles

The bacterial strain of *Shigella flexneri* 2a used for the antigen extraction was isolated from a patient in the Hospital of Navarra in Pamplona, Spain, and the OMVs were extracted at the Department of Microbiology and Parasitology of the University of Navarra.

Brief description of the method

To obtain the OMVs, the bacterial culture had to be inactivated by a solution of 6 mM binary ethylenimine and 0.06% formaldehyde. The culture was then centrifuged and pellet which contained cells was removed. Supernatant with OMVs was purified first by filtration through a 0.22 μm filter to remove large impurities such as whole cells, and then passed through a tangential filtration unit containing a 300 kDa membrane. The retenant was ultracentrifuged and pellet containing purified OMVs was resuspended in water and lyophilized.

4.2 Outer membrane vesicles characterization

Before encapsulating the bacterial antigen into our nanoparticles, various characteristics were studied:

4.2.1 Size and morphology

Morphology and size of the vesicles were evaluated by transmission electron microscopy at the Department of Histology and Pathological Anatomy of the University of Navarra. The sample was diluted in phosphate-buffer saline (PBS) and the suspension (50 μg/ml) was dropped on a 300 mesh copper grid. Two percent Uranyl acetate was then used for negative staining.

4.2.2 Z-potential

To determine the surface charge and gather information about stability of the antigen suspension, Z-potential was measured by ZetaPlus Analyzer. The method of measurment was based on electrophoretic light scattering. In an electric field, particles with different chargers migrate in opposite directions and their mobility (depending on their Z-potential) can be measured by scattering light through the sample.

Procedure OMVs were fist sonicated in distilled water (5 W, 1 min) in order to create a homogenious suspension and Z-potential was measured by the ZetaPlus Analyzer using a palladium electrode in the presence of KCl.

4.2.3 Quantification of proteins

In order to characterize the antigen chemically, the amount of protein was measured by Lowry protein assay. The method is based on the Biuret reaction, in which copper bonds to proteins under alkalic conditions, it is reduced to monovalent ions. Then it reacts with Folin reagent reducing phosphomolybdotungstate to heteropolymolybdenum blue. The reaction results in blue color, which is due to the tyrosine and tryptophan content.

Procedure Alkalic solution was mixed with solution containing Cu^{2+} ions, and the mixture was added to our samples. After 15 min of incubation and 5 min of boiling, Folin–Ciocalteau reagent was added and the samples were incubated for another 60 min. Finally the absorbance was measured by spectrophotometer at 750 nm, using bovine serum albumine (BSA) as a standard. *(See Appendix A)*

4.2.4 Quantification of lipopolysaccharide

Another chemical characterization includes determining the amount of LPS. The method is based on detection of 2-keto-3-deoxyoctonate (KDO), a sugar exclusively found in bacterial LPS. In this method, periodic acid oxidises terminal glycol groups of KDO while releasing formaldehyde, which can be detected by spectrophotometry after reaction with thiobarbituric acid.

Procedure H_2SO_4 and HIO_4 were added to the samples. The oxidation was performed for 20 min at room temperature (RT) and stopped by $NaAsO_2$ in HCl, which degrades excess of the periodic acid (can be seen as a disappearance of brown colour). A freshly prepared aquaeous solution of thiobarbituric acid was then added, the mixture was boiled for 15 min and after adding dimethylsulfoxide (DMSO) the sample was read by spectrophotometer at 540 nm, using purified *Shigella flexneri* 2a LPS as a standard. *(See Appendix A)*

4.2.5 Proteic and lipopolysaccharide profile

SDS-PAGE was subsequently performed to reveal the proteic and LPS profile. The method is based on electrophoresis in the presence of sodium docecylsulphate (SDS), where the proteins and LPS subunits are separated according to their molecular weight and then visualised by various methods.

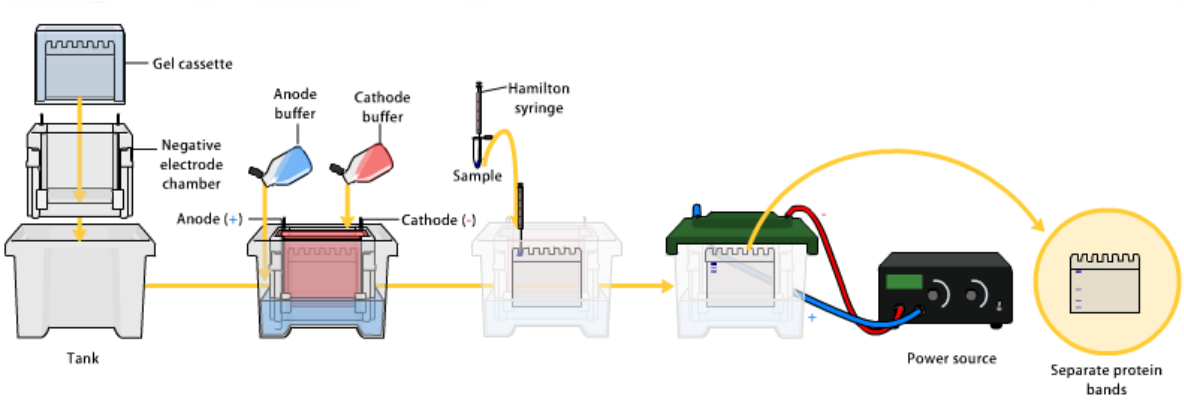


Figure 5: Polyacrylamide gel electrophoresis. Polyacrylamide gel is placed in a tank containing anode (+), cathode (-) and a buffer. After loading proteic samples (treated with sodium dodecylsuphate), electric field is applied, which causes proteins to separate according to their molecular weight. Taken from wikipedia.org [38]

Procedure the sample was resuspended in sample buffer, containing sodium dodecylsulphate as a detergent and β-mercaptoethanol as a reducing agent, boiled for 5 min and loaded on a gel of 12% acrylamide. The gel ran at 200 V for 50 min in the presence

of 3-(N-morpholino)propanesulfonic acid (MOPS) buffer. Coomassie Blue was used to reveal the protein bands while silver staining was used for LPS. *(See Appendix A)*

4.3 Nanoparticles preparation

For further experiments four types of NPs were prepared, all based on protein P which naturally occurrs in food. Alongside simple proteic particles, we also decorated the particles with two main compounds, Compound A and Compound B (the nature of any of the compounds cannot be described due to confidential issues). Their ability to modify surface properties is studied. This resulted in 4 basic types of NPs:

1. P-empty (Proteic NPs)
2. PA-empty (Proteic NPs coated with Compound A)
3. PB-empty (Proteic NPs coated with Compound B)
4. PAB-empty (Proteic NPs coated with Conjugate of AB)

All these types of NPs served as carriers for loading the OMVs, which resulted in second set of NPs:

5. P-OMV
6. PA-OMV
7. PB-OMV
8. PAB-OMV

4.3.1 Preparation of empty nanoparticles

The preparation of our NPs was based on Protein P's solubility in 60% ethanol and insolubility in water. Protein P was solubilized in 60% ethanol under a magnetic stirrer together with a basic amino acid, which modifies the final nanoparticle's surface properties, particularly Z-potential. By adding water to the solution with a constant flow, nanoparticles were formed, which was indicated by a change in the transparency of the liquid.

In the case of preparing coated nanoparticles, corresponding coating compounds solubilized in water were added to the suspension.

After 30 min of incubation, the suspension had to be passed through a concentrator containing a 50 kDa membrane filter in order to remove impurities, such as unbound amino acid or protein residues. Mannitol dissolved in water was subsequently added to

the suspension in order to facilitate the spray drying process. The suspension was then passed through the spray dryer with temperature set to 96 °C. *(See Appendix B)*

4.3.2 Encapsulation of bacterial antigen into nanoparticles

Preparation of OMV-loaded NPs is the same as preparation of empty ones with one additional step. A homogenious suspension of OMVs in water has to be prepared first where OMVs are vortexed and then sonicated (5 W, 1 min) in order to break down the aggregates. Then, this suspension is added to the dissolved Protein P together with water phase (as described in section 4.3.1). *(See Appendix B)*

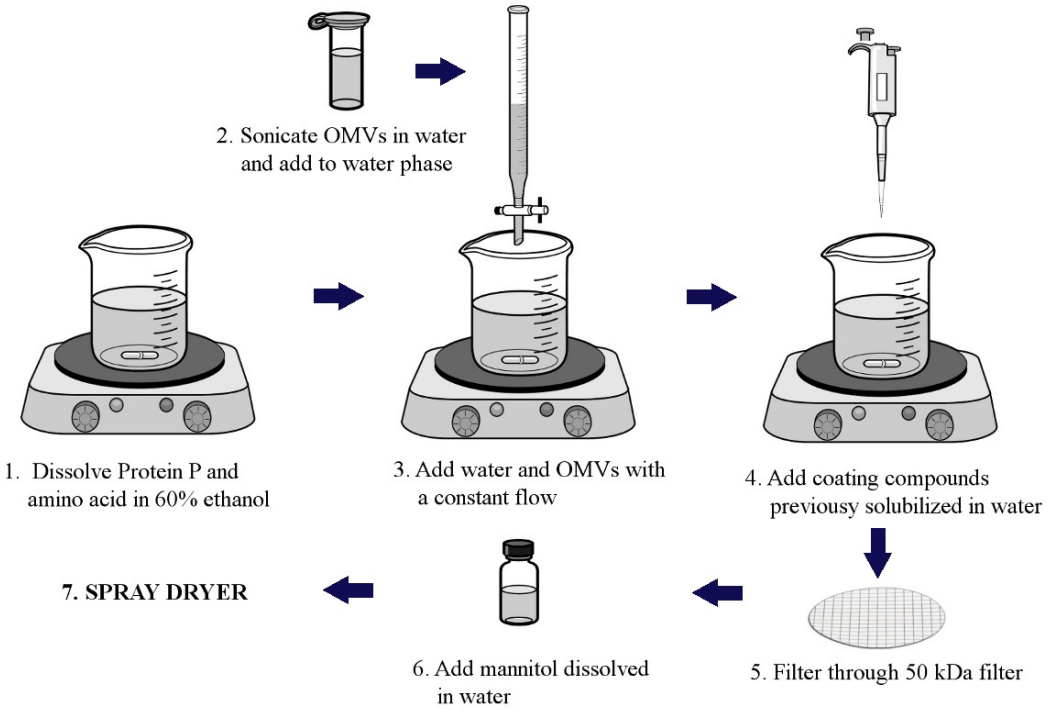


Figure 6: Preparation of nanoparticles. 1. Protein P and basic amino acid are dissolved in 60% ethanol. 2. In case of preparing OMV-loaded NPs, OMVs are sonicated and added in the next step. 3. Water phase is poured into the formulation with a constant flow. 4. Previously solubilized conjugate is added, and the suspension is stirred for 30 min. 5. The formulation is passed through a 50 kDa membrane filter. 6. Finally, mannitol is added in order to facilitate the last step of the preparation: 7. The spray drying process.

4.3.3 Size and Z-potential characterization

Size and Z-potential were measured immediately after preparation as primary characteristics and also as initial indicators of whether the preparation process was successful.

The measurement was based on dynamic light scattering of a suspension of NPs in water (for measuring size) or in a solution containing KCl (for Z-potential).

4.4 Detecting OMVs on the nanoparticles surface

An important part of the experiments was to demonstrate the presence of OMVs in the final product. Firstly, we determined the presence of OMVs on the nanoparticle surface. This might play an important role in the final effect as NPs containing OMVs on their surface would make the antigen more easily accessible to the immune cells even before the NP's degradation.

NPs possibly containing OMVs on their surface therefore had to be separated from the rest of the formulation (4.4.1) and these separated particles were examined by SDS-PAGE (section 4.4.2). Despite this, simple proteic profiles of those particles were not sufficient proof as it does not show whether the revealed proteins have been modified during the preparation process and are able to induce desired immune response. In this case it was important to confirm that the proteins shown by SDS-PAGE are still antigenic. This is why Western Blot was used in the end (section 4.4.3).

4.4.1 Separating the nanoparticles with exposed antigen

For this experiment we used the Dynabeads® Protein G Kit, containing magnetic beads with immunoglobulin-binding Protein G, linked to their surface. This protein is able to bind to the Fc-region of antibodies, creating a complex which can be separated by a magnet.

If we therefore incubate our NPs with specific antibodies, they are able to bind only to the accessible antigen, in other words only to the NPs carrying antigen on their surface. This complex can be separated using Dynabeads® Protein G and subsequently disentangled by a reducing agent. If we detect the presence of NPs (OMVs or Protein P) in the separated sample, we can assume the antigen was accessible and therefore not fully covered by the particle. The result can then be compared to empty NPs, which contain no antigen at all, and are therefore not affected by this procedure.

Procedure During the procedure, NPs were incubated with a serum containing specific antibodies against *Shigella flexneri*. After 60 min of constant agitation at 37 °C, the mixture was incubated with Dynabeads® Protein G for 20 min. The particles were then separated by placing them on a magnetic tube, where the beads migrated towards the magnet and supernatant was removed. The beads were washed by PBS-Tween in

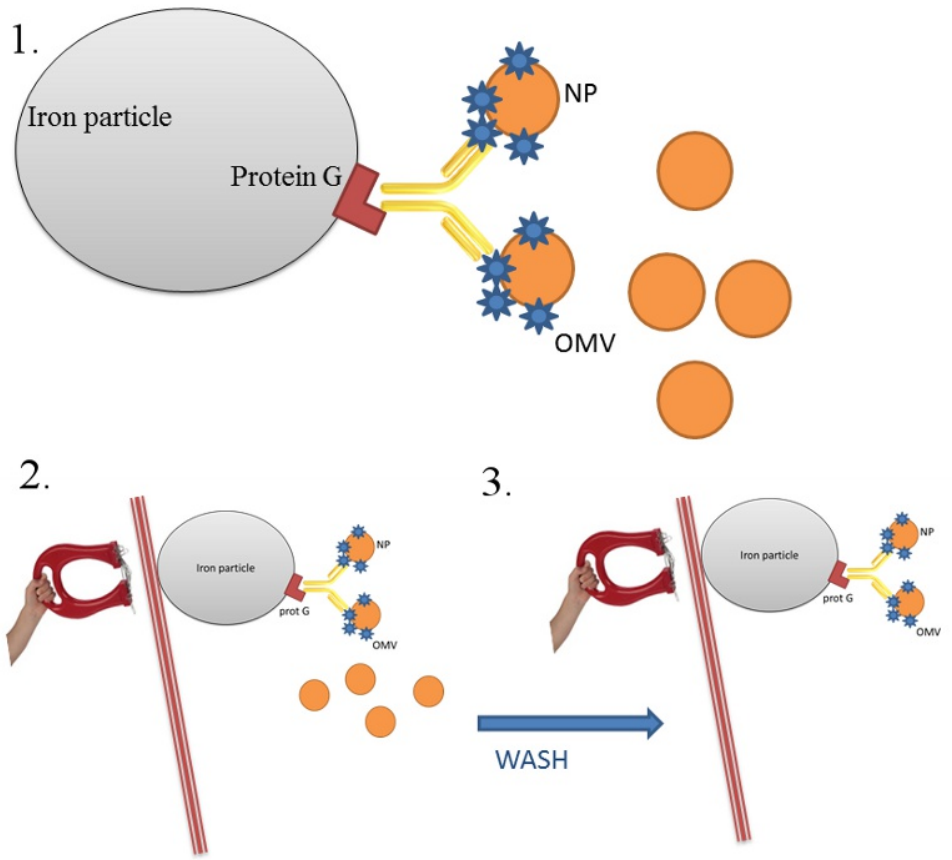


Figure 7: Determination of Outer membrane vesicles on the nanoparticles surface. 1. Nanoparticles are incubated with specific antibodies. Dynabeads® Protein G is added to the mixture and bind to the complex antibodies-antigen. 2. Magnet is used to separate the whole complex from supernatant. 3. By this method, particles containing OMVs on their surface are separated retained by the magnet, while particles without OMVs are washed away.

order to remove the excess of antibodies or remaining NPs. The same experiment was conducted with empty NPs which were later used as a control. *(See Appendix C)*

4.4.2 Proteic profile of separated particles

In order to characterize the complex obtained in the previous experiment (4.4.1), we had to resolve the complex and remove the magnetic parts together with Protein G. We achieved this by using a reducing agent (in our case Sample buffer containing β-mercaptoethanol). Finally, the samples were loaded on SDS-PAGE and the gel was stained by Coomassie Blue in order to reveal their proteic profile. *(See Appendix C)*

4.4.3 Antigenicity of separated particles

While the SDS-PAGE reveals the proteic profile, we still have to confirm that revealed proteins are still antigenic. This can be achieved by using the Western Blot method.

Western Blot is an analytical technique used for detection of specific proteins, in our case antigenic proteins of OMVs. After protein separation by electrophoresis, the proteins are transferred to a polyvinylidene difluoride (PVDF) or nitrocellulose membrane using an electric current. The membrane is incubated first with primary antibodies, which are able to bind directly to the targeted protein. Secondly, the membrane is exposed to species-specific secondary antibody, which target the primary antibody. The secondary antibody is linked to a reporter enzyme, which allows visualisation. In our case, colorimetric detection was performed, based on reaction with hydrogen peroxide (as a main substrate to our reporter enzyme, peroxidase) and chloronaphthol, which is subsequently reduced to a purple-colored precipitate visible to the eye. This way, the desired proteins can be observed as purple bands.

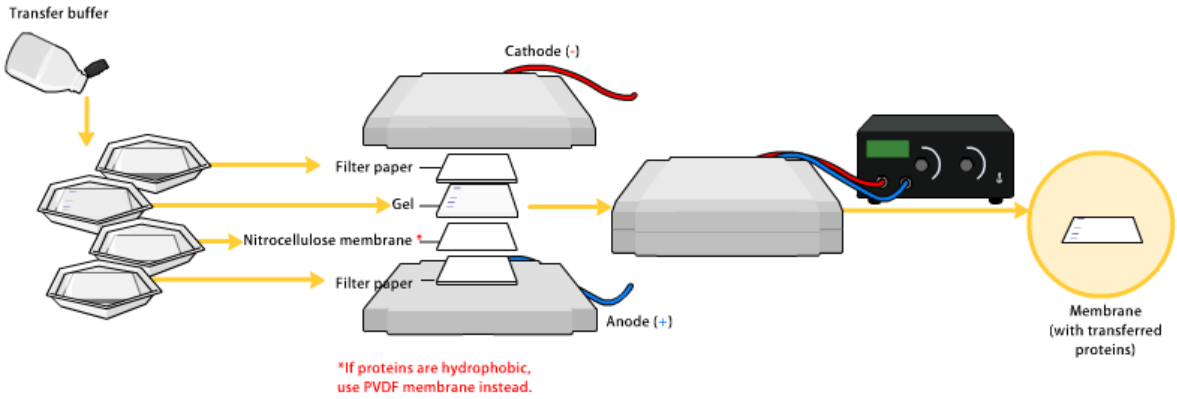


Figure 8: Western Blot transfer. Pieces of filter paper, gel and nitrocellulose membrane are soaked in a transfer buffer. By applying the electric voltage, proteins are transferred to the membrane. Taken from wikipedia.org [39]

Procedure After performing SDS-PAGE, the gel proteins were transferred to a nitrocellulose membrane, using an electric voltage (8 V, 30 min) in the presence of transfer buffer. The membrane needed to be blocked in 5% solution of milk in a transfer buffer in order to prevent non-specific binding of the antibodies to the membrane. The membrane was then incubated for 4 h with the primary antibodies (present in the serum from rabbit hyperimmunized with *Shigella flexneri*), diluted 1:100 in the blocking solution. After washing with PBS-Tween, the membrane was incubated for 1 h with secondary antibodies (Goat anti-Rabbit IgG) conjugated with horseradish peroxidase and diluted 1:1000 in the blocking solution. After another washing with PBS-Tween

the membrane was treated with the revealing solution, containing α-chloronafrol and hydrogen peroxide as the main active ingredients. *(See Appendix C)*

4.5 Dissolving the nanoparticles

While the previous experiments revealed antigen on the surface, it was also necessary to determine the total amount of OMVs in the formulation. By finding a method to determine the total OMVs, we can see how efficient the encapsulation process is and how much antigen is lost during the preparation. Besides, by comparing the total amount of OMVs and the amount of OMVs on the surface, we can eventually demonstrate how much antigen is trapped inside and covered by the nanoparticle:

$$OMVs\ (total) - OMVs\ (surface) = OMVs\ (inside)$$

Determination of total OMVs could be achieved by SDS-PAGE, but first it was necessary to dissolve the NPs and remove all the protein P in order to prevent any interference. The possible presence of free (non-encapsulated) OMVs in our formulation also had to be taken into account.

A method of dissolving the NPs was developed at the Department of Pharmaceutical Technology and involved use of 60% ethanol, which is supposed to dissolve the protein, followed by centrifugation at 27.000 g, which sedimented the non-dissolved material. This method, however, was developed for nanoparticles loaded with other, non-proteic substances. If used with our NPs, significant interference occurred on SDS-PAGE, caused by proteic nanoparticle residues. For this reason, a more effective solvent (section 4.5.2) and an optimal centrifugal force (section 4.5.1) had to be determined first.

4.5.1 Determination of optimal centrifugal force

The first step of the dissolution method is to resuspend NPs in water in order to dissolve the mannitol present in the formulation, which can be removed together with the supernatant after first centrifugation. In this step, we also needed to separate our particles with encapsulated antigen from any free, non-encapsulated antigen. Thus the objective of this experiment was to define the lowest relative centrifugal force (RCF) at which the NPs will sediment and free OMVs will not.

The choice of method was empirical and based on the equipment available. We prepared two samples, free antigen and PAB-empty nanoparticles, both of which were labelled with fluorescent colours, and we centrifuged them at various RCFs. Then we measured

and compared the fluorescence of the obtained supernatant using flow cytometry. By creating a very narrow flow, in which particles pass individually in front of a laser, the flow cytometry techinque can detect, count and characterize the fluorescently labelled cells. Besides this, the fluorescence of the samples can be measured, which we could use to indicate the presence of OMVs or particles present in the supernatant.

Procedure Our samples were labelled by fluorescent colours: OMVs labelled with dioctadecyloxacarbocyanine perchlorate (DiO) and PAB-empty labelled with Lumogen® F Red 300. *(See Appendix D)*

The labelled samples were resuspended in water and then centrifuged at the chosen centrifugal forces:

1. 0 g. (positive control)
2. 10.000 g.
3. 15.000 g.
4. 30.000 g.

Finally the supernatants were removed and their fluorescence measured and compared.

4.5.2 Finding an optimal solvent

60% ethanol, used for dissolving protein P, did not seem to be sufficient for our experiments. It was necessary to find a more effective solvent, or combination of solvents, by using the available equipment. The experiment was based on simple observation. Retaining the concepts of the original method, we resuspended our NPs in various solvents; centrifuged and visually compared size, colour and stability of each pellet. Such a method, though not precise, was simple, fast and served our purpose sufficiently.

Procedure PAB-OMV NPs were resuspended in water (10 mg/ml) in order to dissolve mannitol present in the formulation, then centrifuged at 10.000 g, RCF determined in previous step (4.5.1), and supernatants containing mannitol and free OMVs were removed. Resulting pellets that we obtained were resuspended in tested solvents, chosen after consulting relevant literature:

1. Ethanol 60%
2. Ethanol 70%
3. Ethanol 85%
4. Ethanol 95%

5. Isopropanol 85%
6. Acetone 70%

After a second centrifugation at 10.000 g the pellets were visually observed and their size and color were compared. At the end, DMSO 20% was added as a strong solvent, in order to see its effect on the remaining pellets.

4.6 Revealing the encapsulated antigen

By implementing the results obtained in the previous experiments (section 4.5) to the original dissolution method, a new protocol was designed. All of our formulations were then put through the process; OMV-loaded NPs were dissolved in order to obtain the encapsulated antigen and empty NPs served as a control to see whether the dissolving process was successful.

Procedure Our NPs were resuspended in water and centrifuged. The supernatant containing mannitol and free OMVs was removed and a pellet containing NPs resuspended in 70% ethanol, which partly dissolved protein P. Another centrifugation resulted in pellet containing OMVs and proteic residues. This pellet was resuspended in a mixture of 20% DMSO in 85% ethanol and the last centrifugation was performed. After removing the supernatant, we obtained the final pellet, which was used for further experiments. *(See Appendix E)*

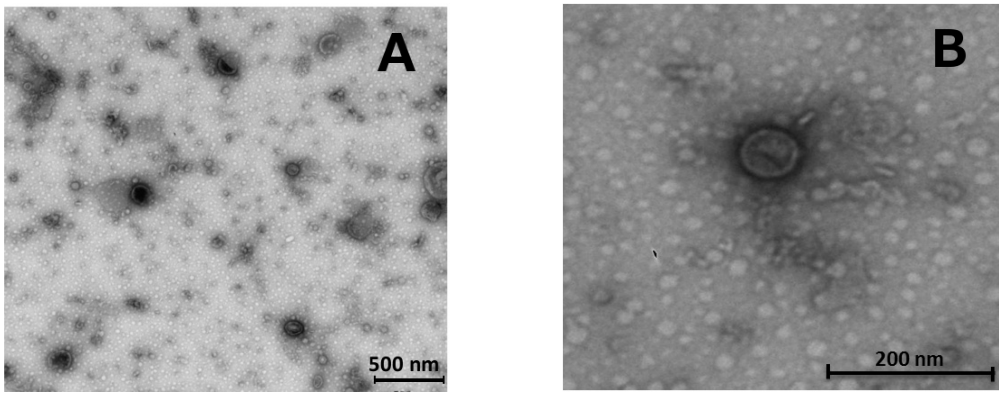
4.6.1 Proteic and LPS profile

The pellet obtained in the previous step was loaded on the SDS-PAGE in order to reveal its proteic and LPS profile. Two types of staining were subsequently used: Coomassie Blue for revealing the proteins and Silver staining for revealing the LPS. Free OMVs were used as a control. *(See Appendix E)*

5. Results

5.1 Outer membrane vesicles characterization

Electron microscopy The electron microscopy shows OMVs as spherical vesicles with a wide range of diameters of 15-150 nm (see Fig. 9).



(a) (b)

Figure 9: Transmission electron microscopy of free OMVs: electron photomicrograph with scale bars indicating 500 nm (A) or 200 nm (B).

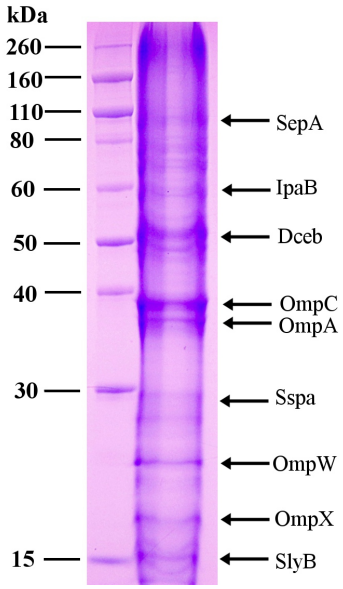
Z-potential Surface charge measured by ZetaPlus Analyzer showed to be negative, with a value of -9.82 ± 0.018 mV (see Tab. 3)

Protein and LPS quantification Protein content determined by the Lowry assay was 32.8 ± 2.8%, whereas LPS content measured by the KDO assay was 39.4 ±8.2% (see Tab. 3)

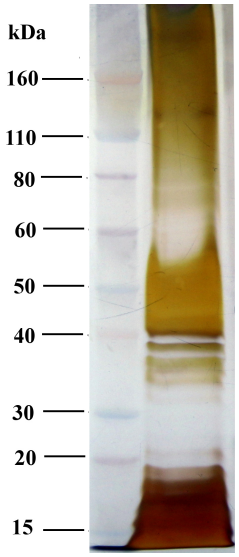
Proteic and LPS profile Proteic and LPS profiles revealed by SDS-PAGE and stained by Coomassie Blue and Silver reagents are shown in Fig. 10. Proteomic analysis of the separated bands identified some proteins which are significant for bacterial virulence, such as SepA (110 kDa), IpaB (62 kDa), Dceb (OmpC (38 kDa), OmpA (34 kDa) Sspa (24 kDa), OmpW (22 kDa), OmpX (18 kDa) and SlyB (15 kDa). The LPS profile revealed by silver staining showed a characteristic ladder pattern in the area between 10 an 40 kDa.

	OMVs
Size	15 - 150 nm
Z-potential	-9.82 ± 0.018 mV
Amount of proteins	30.8 ± 2.8 %
Amount of LPS	39.4 ± 8.2 %

Table 3: Basic characteristics of Outer membrane vesicles. Size, Z-potential and percentual amount of proteins and LPS were measured before encapsulation into nanoparticles.



(a) Proteic profile of free OMVs



(b) LPS profile of free OMVs

Figure 10: Proteic and lipopolysaccharide profile of free Outer membrane vesicles determined by gel electrophoresis. Significant proteins can be found (a) as well as characteristic ladder pattern at the area between 10 an 40 kDa (b).

5.2 Preparation of nanoparticles

All prepared NPs were obtained in a form of beige-white powder. No significant difference in their appearance or color was observed. Tab. 4 shows the basic characteristics of all NP formulations, which were measured straight after preparation.

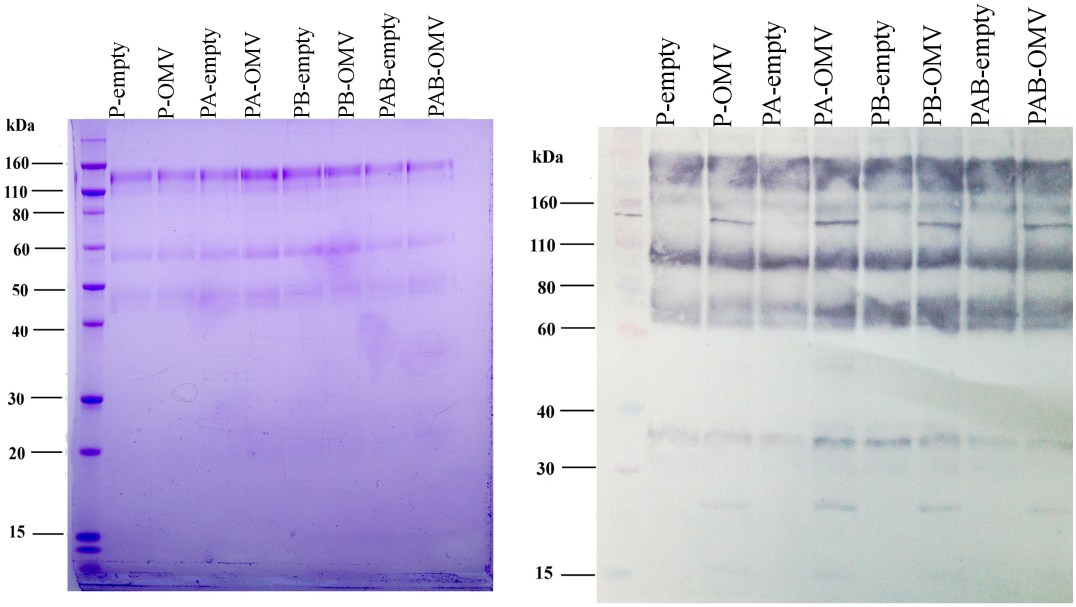
	Yield (%)	Size (nm)	Polydispersity	Z-potential (mV)
P-empty	35.05	223.7 ± 2.1	0.073 ± 0.11	-15.37 ± 0.78
P-OMV	37.07	373.3 ± 3.1	0.274 ± 0.019	-19.30 ± 0.56
PA-empty	47.59	275.0 ± 2.1	0.098 ± 0.014	-33.00 ± 1.04
PA-OMV	46.43	229.5 ± 0.7	0.069 ± 0.021	-30.95 ± 2.06
PB-empty	45.16	222.9 ± 1.6	0.155 ± 0.006	-29.54 ± 0.37
PB-OMV	46.29	299.1 ± 3.0	0.079 ± 0.015	-47.43 ± 0.87
PAB-empty	47.68	262.1 ± 4.4	0.095 ± 0.015	-53.16 ± 1.02
PAB-OMV	46.98	402.1 ± 0.7	0.104 ± 0.024	-33.94 ± 0.44

Table 4: Basic characteristics of nanoparticles. Yield of the reaction, size, polydispersity and Z-potential were measure after NP preparation.

We can notice a slight increase of yields of coated nanoparticles, however, no significant differences among the coating compounds was observed. Polydispersity of all formulations can be considered low in all formulations. Z-potential is negative and again we can observe a decrease in the Z-potential of all coated nanoparticles.

5.3 Detecting antigen on the nanoparticles surface

By using Dynabeads® Protein G Kit, we were able to separate NPs possibly containing antigen on their surface. Those separated samples were loaded on SDS-PAGE and their proteic profile was compared to empty NPs. Differences in the loaded and empty NP profiles were evaluated. For a more specific result, Western Blot was performed. While SDS-PAGE gel shows very weak bands at both empty and loaded NPs, Western Blot reveals clear bands of approx. 150 kDa, which do not occur with the empty formulations (Fig. 11).



(a) Sufrace antigen: proteic profile

(b) Surface antigen: antigenicity

Figure 11: Detection of Outer membrane vesicles on the surface: Electrophoresis gel (a) and Western Blot membrane (b). SDS-PAGE shows no significant difference between empty and loaded nanoparticles. However, after transferring to a nitrocellulose membrane and performing Western Blot, clear bands appear with the loaded samples.

5.4 Revealing the encapsulated antigen

The efficacy of encapsulation is a key factor in the process; however, determining the presence of antigen in the final product through standard and available methods (such as SDS-PAGE) is complicated by the presence of protein P in the nanoparticle. Additionally, the formulation had to be centrifuged and cleared of any free antigen to prevent false positive result.

5.4.1 Finding an optimal centrifugal force

In order to find an optimal centrifugal force for separating the free antigen, empty nanoparticles and OMVs, both fluorescently labelled, were centrifuged: Obtained supernatants were measured by Flow cytometry. In the case of nanoparticles, the number of particles in the supernatant was measured by virtue of fluorescent labelling of the nanoparticles. In the case of OMVs, the fluorescence itself was measured in the supernatant.

Fig. 12 shows the amount of nanoparticles in the supernatant, distributed by size. All measured samples are depicted, a significant difference between non-centrifuged NPs (yellow) and supernatants, obtained after centrifugation at 10.000 g, 15.000 g and 30.000 g, can be observed. From this perspective, the centrifuged samples (blue, green and purple) show an insignificant signal.

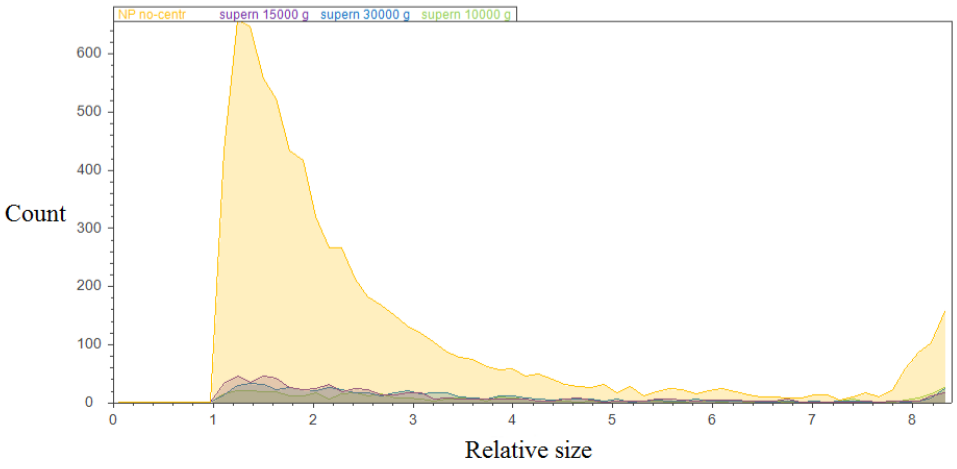


Figure 12: Fluorescence of supernatants retained after centrifugation. Difference between non-centrifuged nanoparticles (yellow) and supernatants obtained after centrifugation at 10.000 g, 15.000 g and 30.000 g can be observed. The centrifuged samples (blue, green and purple) show an insignificant signal.

When looking closely at the centrifuged samples (Fig. 13), even though a slight difference between the signals can be observed, the samples can be considered comparable.

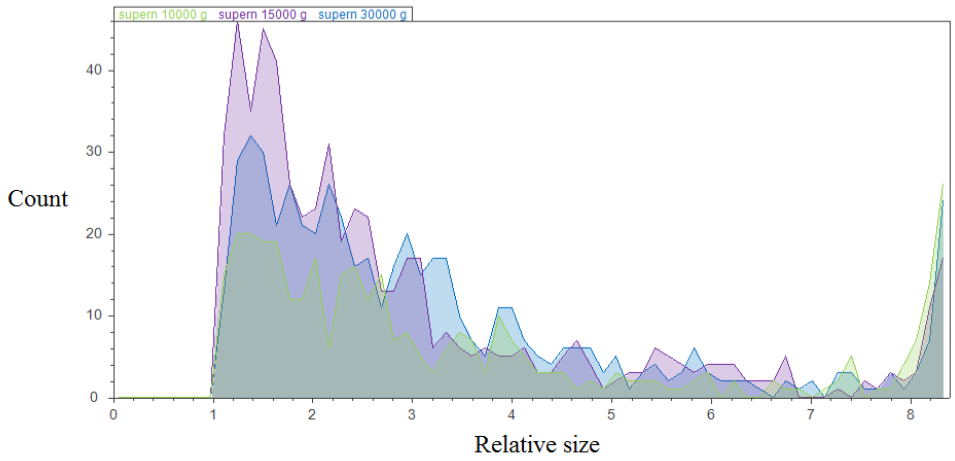


Figure 13: Fluorescence of supernatants after centrifugation: detail. Detail of centrifuged supernatants shows that the fluorescence was the highest in the sample centriguged at 15.000 g, while the lowest was at the sample centrifuged at 10.000 g.

The fluorescently labelled OMVs are shown in Fig. 14. Here again we can compare supernatants after centrifugation with non-centrifuged control.

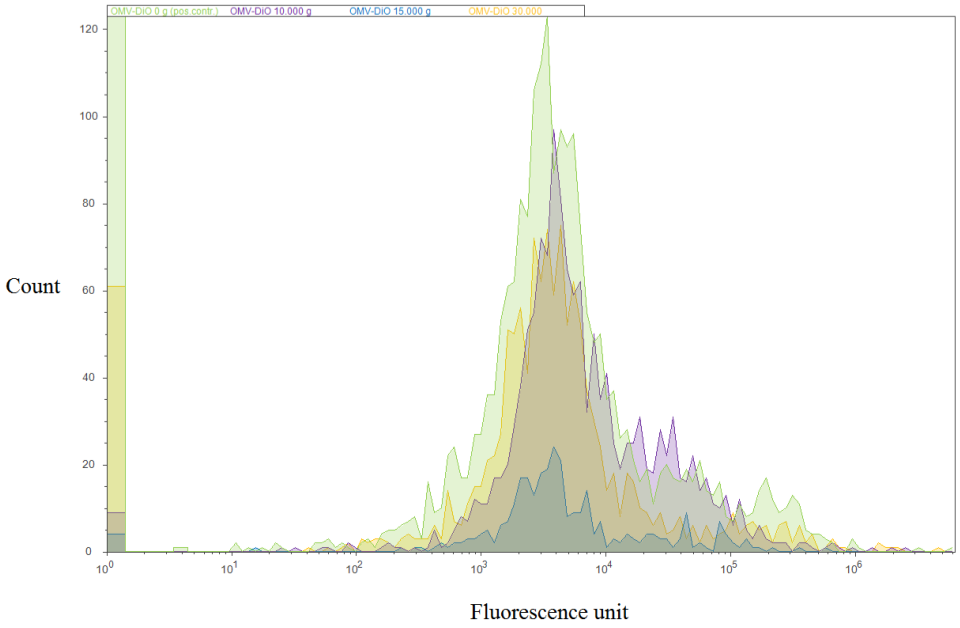


Figure 14: Fluorescence of supernatants after centrifugation of Outer membrane vesicles: compared to the control (0 g), two of the samples after centrifugation (10.000 g and 30.000 g) show a similar fluorescence, the sample after 15.000 g shows the lowest signal.

5.4.2 Finding an optimal solvent

Once we selected the minimal RCF which is able to selectively sediment the NPs, our second goal was to find an optimal solvent for dissolving the NP protein. Thus, OMV-loaded NPs were tested with following solvents:

1. Ethanol 60%
2. Ethanol 70%
3. Ethanol 85%
4. Ethanol 95%
5. Isopropanol 85%
6. Acetone 70%

The efficacy of these solvents was determined after centrifugation. While the dissolved protein was not sedimented, non-dissolved parts created a pellet when centrifuged. By simple visual examination, we compared the size and colour of the pellets in each sample.

Although none of the solvents were able to completely dissolve the nanoparticles, differences among the pellets were observed. Of the tested solvents, ethanol 70% and ethanol 85% elicited (under described circumstances) the smallest pellets. These were comparable in size, but different in color. While 70% ethanol created a small bright yellow pellet, the pellet of 85% ethanol was white. In the second part of the experiment, DMSO 20% was added in order to increase the solvent capacity. After vortexing the samples, we examined the transparency of the liquids. While most of the samples still contained non-dissolved aggregations of NPs, samples with ethanol 70% and ethanol 85% created clear liquid, which indicated complete dissolution of the polymeric protein NP.

5.4.3 Proteic profile of revealed antigen

After finding an optimal RCF and efficient solvents, all our NPs were dissolved using the new protocol, which included use of ethanol 70%, ethanol 80%, DMSO in a combination with centrifugation at 10.000 g. The efficiency of the OMV encapsulation could be determined by SDS-PAGE. While none of the empty NPs show any bands, the OMV-loaded NPs show clear bands, comparable to the control - free OMVs. The molecular weight of approx. 38 kDa, 34 kDa, 22 kDa and 18 kDa indicates presence of the main OMV proteins: OmpC, OmpA, OmpW and OmpX. (Fig. 15)

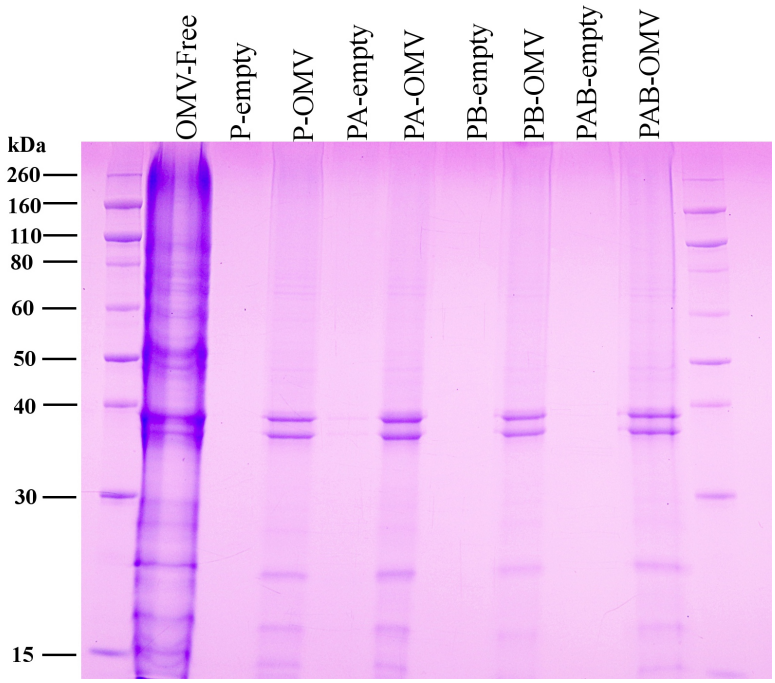


Figure 15: Proteic profile of revealed Outer membrane vesicles after NP breaking: the efficiency of OMV encapsulation was determined by SDS-PAGE. The gel shows protein bands only in the OMV-loaded samples, with molecular weight comparable to the control (free OMVs).

The same dissolving procedure was performed again to examine LPS profile of the encapsulated OMVs by SDS-PAGE. By comparing these samples with the free OMVs, we can see a clear profile at P-OMV, PB-OMV and PAB-OMV NPs, while the signal of PB-OMV is notably weaker. However, when focusing on the characteristic ladder pattern of LPS, which can be seen between 15 - 30 kDa, all the samples show a comparable result. Empty NPs (included as a control of the staining method and dissolution efficiency) showed no bands with the exception of a very weak coloured stripe of approx. 30 kDa. (Fig. 16)

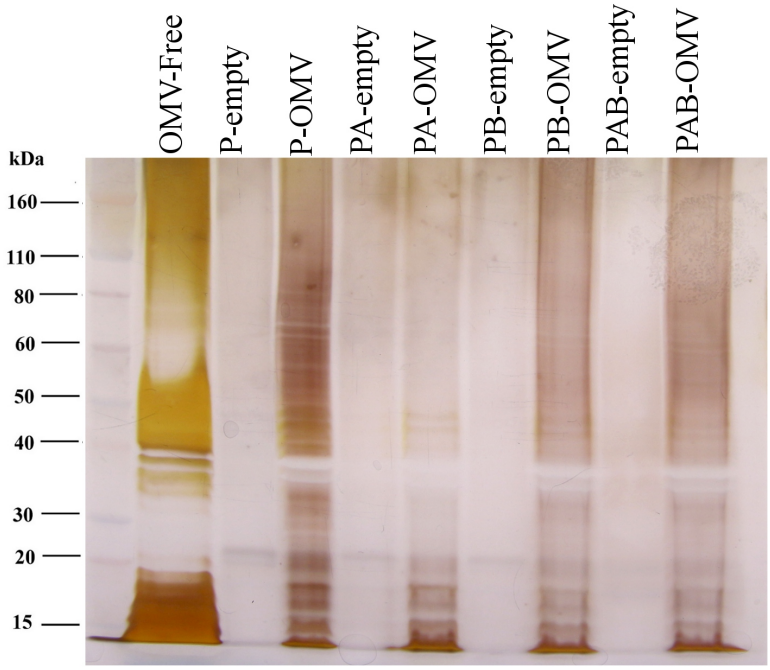


Figure 16: Lipopolysaccharide profile of revealed Outer membrane vesicle after nanoparticle dissolving. Even though the signal of PB-OMV NPs is comparably weaker than other NPs, the characteristic ladder pattern in the lower part of the gel can be observed at all examined samples.

6. Discussion

In 1991 the World Health Organization stated that developing safe and effective *Shigella* vaccines was one of the research priorities [40]. The goal has not yet been achieved and we still have no safe and effective *Shigella* vaccine on the market. As it turns out, the major obstacles are hinted by the words "safe and effective". While studies on live attenuated vaccines demonstrated the ability to provide high protection, adverse effects and other major drawbacks (such as need for multiple primary doses and annual boosters) prevented large-scale use [15]. Non-living vaccines, on the other hand, nowadays represent the safest option for immunization. However, the route of administration has to be taken into account as well. To protect against *Shigella*, both mucosal and systemic immunity need to be induced, for which the mucosal route of administration shows a higher potential [16]. We also have to consider the target population, where the mucosal vaccination seems advantageous as well, there is no need for needles or healthcare professionals and it is generally more suitable for massive use in developing countries, especially for children. To sum up, a non-living, single dose, mucosal vaccine seems to be the optimal solution.

Our approach is based on using Outer membrane vesicles (OMVs) as a subunit antigen. These vesicles, released by G-negative bacteria, are formed from the bacterial membrane, contain many membrane proteins as well as lipopolysaccharide and are an important virulence factor. In previous studies, a single dose of *Shigella* OMVs administered mucosally showed a protective effect on mice [1].

After extracting the OMVs, we examined the chemical composition and found that they contained 32.8% of proteins and 39.4% of LPS. This confirms our assumption that OMVs contained a large amount of LPS originating from the bacterial outer membrane. When examining the LPS profile of our OMVs, we were able to see a typical ladder pattern of LPS. This pattern is identical among different species of Gram-negative bacteria [41], yet the result we obtained is comparable to the results from previous studies with *Shigella flexneri* [42]. Thus, the presence of LPS was confirmed and the profile could function as a control for our experiments, namely determination of LPS in the final NPs.

Apart from LPS, a considerable amount of membrane proteins were also present. The

proteic profile and further proteomic study revealed proteins such as OmpX, OmpC, OmpA, IpaC, Dceb, IpaB or SepA. These proteins are involved in the pathogenesis of the bacterial infection. For instance it is suggested SepA might be involved in invasion and destruction of the host intestinal epithelium [43]. Similarly, previous studies showed the absence of OmpC causes a reduction of virulence [44]. OmpA is considered one of the most immunodominant antigens in the outer membrane of Gram-negative bacteria. It has many characteristics desired of a vaccine candidate, as it boosts induction of IgG and IgA in both the systemic and mucosal compartments and also activates Th_1 cells [45]. IpaB and IpaB are required for efficient intercellular dissemination of *Shigella* and are required for the escape of *Shigella* from the phagosome in infected macrophages [46]. These results support our assumption that OMVs posses immunomodulation properties required for their use as an antigen in a *Shigella* vaccine.

However, the mucosally administered OMVs face several problems. For instance, the passage through the gastrointestinal tract, absorption and acidic pH of the stomach would complicate reaching immune system. To ensure protection and effective delivery of the antigen to the immune cells, a suitable adjuvant would be needed. Among many different adjuvants, nanoparticles showed promising results, as they are capable of protecting the antigen from degradation, and could deliver and release the antigen at the desired regions. The adjuvanticity and the immune response induced by the nanoparticles is influenced by their physical characteristics, namely, size, superficial charge, amount of loaded antigen and the particle's ability to present or release this antigen [47]. These characteristics are therefore discussed further.

Nanoparticles based on Protein P are currently studied at the University of Navarra. Apart from that, two various coating compounds were used in the preparation. As a result, four types of unloaded and four types of OMV-loaded NPs were prepared, ranging from 222.9 ± 1.6 nm to 402.1 ± 0.7 nm in size. Generally, the size of particles can determine the mechanism for their uptake by macrophages and dendritic cells. It is believed that internalization of smaller particles with such size range can occur through macropinocytosis [48]. As for Z-potential, our particles ranged from -53.94 ± 0.44 to -15.38 ± 0.78 mV. Negative charge of the particles was a desired feature, as particles bearing cationic or anionic surface charges have been shown to be more attractive to phagocytes than neutral particles of the same size [49].

At this point, the effect of the coatings was also observed. First of all, we observed an approx. 10% increase in yield of all of the coated NPs compared to non-coated ones. Second of all, a significant decrease in the Z-potential of the coated NPs was observed. These two parameters might indicate that the coating process was successful.

Further evaluation was focused on the encapsulation process and its efficacy. It was important to take note of the differences in Z-potential between empty and OMV-loaded NPs of the same type. As shown in Table 4, two of our formulations (P-NPs and PB-NPs) show a decrease of Z-potential after loading with antigen. Our OMVs show a negative Z-potential (-9.82 mV) and this result might indicate that OMVs are at least partially exposed on the NPs' surface, however this hypothesis had to be confirmed in the following experiments. The other two formulations (PA-NPs and PAB-NPs) show an increase in Z-potential of the loaded NPs when compared to the empty ones. This could potentionally indicate that none of the antigen is exposed on the NPs' surface, however, it is important to mention that results obtained by measuring PAB-NPs in previous experiments at the department differ [50]. Therefore we suggest further examination.

In our experiments we focused not only on detecting the presence of antigen, but also on its localization, as it can be either encapsulated in the NPs or exposed on the surface. By encapsulating the antigen, we can provide it with a protection from degradation or, in case the antigen induces a short-lived immune response, achieve a more efficient delivery. On the other hand, NPs covered by antigen on their surface can present the antigen to the immune cells in a similar way to how it would be presented by the pathogen itself.

In the next step of the experiments we focused particularly on whether the OMVs were exposed on the surface. What we found was a significant difference between empty and loaded NPs, both of which were treated by the Dynabeads® Protein G procedure. When looking at the resulting SDS-PAGE, no difference between empty and loaded NPs was observed. What is more, the bands were very weak with all of our samples. However, after conducting a Western Blot using specific anti-*Shigella* AB, new bands occur. It is important to mention that specific anti-*Shigella* AB were also used during the Dynabeads® ProteinG separation process, which explains a set of clear bands occuring within all our NPs, empty and loaded. When ignoring these bands, we can see differences between the empty and loaded NPs at approx. 80 kDa, which indicated presence of an antigenic protein. Its nature, however, remains unclear.

Further steps were focused on total amount of antigen captured by the particle. Using SDS-PAGE we encountered a significant problem - both OMVs and nanoparticles contained proteins, which were impossible to distinguish by SDS-PAGE. In addition, the formulation had to be cleared from any free OMVs possibly present in the formulation. An optimal relative centrifugal force was chosen by comparing the fluorescence of centrifuged samples. The experiment revealed that our nanoparticles significantly

sedimented even at the lowest chosen RCF. This is why 10.000 g was chosen for further experiments.

Next the nanoparticle protein had to be dissolved. Many chemicals suitable for dissolving protein P are described in literature, however, none of the tested solvents was able to dissolve the protein completely. This is explained by the fact that protein P is actually a mixture of proteins varying in molecular size and solubility. The percentage of each protein type can vary according to the method used for protein extraction, in other words, composition of protein P can vary and there is no universal solvent (source cannot be mentioned due to confidential issues). This was confirmed by our observations, as a combination of more solvents (namely ethanol 70 %, ethanol 85 % and dimethylsulfoxide) was needed. These mentioned solvents were evaluated as optimal for our particular protein P and they were therefore used for further experiments.

When applying the new method of dissolving our nanoparticles, we were able to eliminate the disturbance of proteic cover and reveal important OMV proteins which were present in the formulation. As mentioned above, these proteins (especially OmpC and OmpA) are the most immunodominant proteins of OMVs, however, it remains unclear whether these proteins are covered by the NP or if they are at least partially exposed on the surface. In addition, even though we could detect these proteins in the final product, we cannot be sure they have not been altered during the preparation process. A Western Blot will be a suitable method to make sure the antigengenicity of these proteins is preserved. When comparing all four NP types, we can see no significant difference in the occurring proteic bands. This observation is similar to the one we had in section 4.4 (Detecting antigen on the nanoparticles surface); all our formulation seem to contain a comparable amount of OMV proteins.

In the last step, we evaluated the presence of LPS after using the same dissolving method. As mentioned above, LPS profile is characterized by a typical pattern in the area between 15 and 30 kDa. Even though the gel revealed slight differences among the NP types, the typical pattern was preserved in all our formulations and it was comparable to the control (free OMVs). Here again, we can conclude that the amount of LPS is comparable in all our NPs. To sum up, our results showed that all our NP types contained a comparable amount of OMVs, in other words, we couldn't see any effect of the coating on the OMV encapsulation. Such observation is essential for further research, which includes cellular uptake studies. Thus, if any differences will be observed at this level, we can suggest that they will not be caused by different amount of antigen in the formulations.

7. Conclusions

The results of this work can be summarized in the following points:

1. Nanoparticles based on Protein P are capable of carrying important immunogenic structures of *Shigella flexneri*
2. These structures are present on the nanoparticles surface
3. The amount of *Shigella* proteins and lipopolysaccharide is comparable in all of the formulations. Thus, coating the nanoparticles with Compound A and Compound B has no significant effect on the amount of the carried antigen

These results serve as a primary evaluation of the new nanoparticle types and support their further examination. In the following experiments, I would suggest to:

- Perform Western Blot using the revealed encapsulated antigen in order to make sure the proteins have not been changed or damaged during the preparation process and are still able to bind with specific antibodies
- Evaluate the effect of the new dissolving method on the encapsulated OMVs. This step is essential for measuring the exact amount of OMVs present in the nanoparticles
- Using previous step, evaluate the amount of OMVs inside the nanoparticle only
- Determine the ability of the NPs to release the entrapped antigen in physiologic environment

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Appendices

A Chemical characterization of OMVs

Lowry protein assay

Reagents

Solution A: 0.4% NaOH, 0.2% Na_2CO_3, 0.16% sodium tartrate, 2% SDS in H_2Od
Solution B: 0.4% $CuSO_4$. 5 H_2O in H_2Od
Folin-Ciocalteus's reagent

Sample: OMVs 3 mg/ml
Standard: BSA 1% solution in PBS-Tween

Procedure

- BSA standard protein solutions (in duplicates) were prepared from 1% BSA as indicated below:

	1.	2.	3.	4.	blank
BSA	100 μl	50 μl	25 μl	12.5 μl	0 μl
H_2Od	100 μl	150 μl	175 μl	187.5 μl	200 μl

- Samples were prepared in triplicate by taking 200 μl of OMVs (3 mg/ml) in separate tubes
- One ml of Solution B was added to 100 mL of Solution A, 1 ml of the mixture was added to the all the samples
- After 15 min of incubation and 5 min of boiling, 100 μl of Folin-Ciocalteau reagent was added and the samples were incubated for another 60 min
- The absorbance was measured by spectrophotometer at 750 nm

KDO assay

Reagents

H_2SO_4 1.25 mol/l
HIO_4 0.042 mol/l
$NaAsO_2$ 2% in 0.5 M HCl
Thiobarbituric acid 0.3% in H_2Odd
DMSO (pure)

Sample: OMVs 3 mg/ml
Standard: Purified LPS of *Shigella flexneri* 3mg/ml

Procedure

- The standard LPS solutions (in duplicates) were prepared from LPS of *Shigella flexneri* (3mg/ml) as indicated below:

	1.	2.	3.	4.	blank
LPS	200 μl	100 μl	50 μl	25 μl	0 μl
H2Od	0 μl	100 μl	150 μl	175 μl	200 μl

- Samples were prepared in triplicate by taking 200 μl of OMVs (3 mg/ml) in separate tubes
- Twenty μl of $H_2SO_4$was added and the samples were boiled for 20 min
- After 15 min recovery, 250 μl of periodic acid was added
- After 2 min vortex, 500 μl of $NaAsO_2$ was added, the samples turned yellow and the color dissapeared after approx. 2 min
- Two ml of thiobarbituric acid was added and the samples were boiled for 20 min
- One ml of DMSO was added, then the samples were let stand for 1 min in ice and then let recover for approx. 30 min in room temperature until the color turned purple
- The absorbance was measured by spectrophotometer at 552 nm

Proteic and LPS profile

Reagents

PBS
Sample buffer
Coomassie Brilliant Blue
Revealing solution: 5 % isopropanol, 15 % acetic acid in H_2Od

Solution A	50% methanol, 40% acetic acid, 40% water
Solution B	7.5% methanol, 5% acetic acid, 92% water
Developing solution	32 g of Developer in 1 l dionized water (H_2Od)
Stopping solution	5% acetic acid in water

Paraperiodic acid
Silver reagent

Sample: OMVs 1 mg

Procedure

- The sample in duplicate was resuspended in 40 μl of PBS and 20 μl of Sample buffer
- The samples were vortexed properly and boiled for 5 min
- The boiled samples were loaded on 12% acrylamide gel and run for 50 min at 200 V
- For the protein staining, the gel was washed in Coomassie Brilliant Blue for 2 h and then in Revealing solution for 2 h
- For the revealing of LPS, the gel was washed in Solution A for 30 min, then in Solution B for 60 min and then washed with H_2Od for 10 min
- Next, the gel was oxidised for 10 min with a solution of 0.7 % paraperiodic acid in Solution B and washed thoroughly (20 min in H_2Od and 20 min in double dionized water (H_2Odd))
- The silver reagent was then used on the gel for 20 min, followed by washing
- The gel was then soaked in the Developing solution
- After visible brown bands occured, the gel was soaked in the Stopping solution in order to stop the staining reaction

B Nanoparticles preparation

Solubilization of the coating compounds

A Compound A was hydrolysed in H_2O (9.79 mg/ml) under magnetic stirrer for 5 days. Final solution can be stored at RT.

B Compound B was dissolved in H_2O (0.21 mg/ml). Final solution is stored at 4°C.

AB Firstly a conjugate of A and B had to be prepared: 5 g of Compound A was dissolved in 600 ml of ultra pure acetone. 250 mg of Compound B was added and solution was incubated for 3 hours at 40–50°C at constant stirring. Acetone was then evaporated using rotavapor. Solid conjugate obtained after evaporating can be stored at RT.

Before NPs preparation the conjugate was solubilized in water (10 mg/ml) under magnetic stirrer for 5 days. Final solution can be stored at RT.

Preparation of empty NPs

Reagents

Protein P	300 mg
Basic aminoacid	50 mg
Mannitol	600 mg
Ethanol 60%	30 ml
Distilled water	30 ml

For coated NPs :

Compound A/B/AB	750 μl	(previously solubilized)

Procedure

- Protein P and basic aminoacid were dissolved in 30 ml of ethanol 60% under magnetic stirrer
- 30 ml of distilled water was added using a syringe tube in order to create a constant flow
- In case of preparation of coated of NPs a 750 μl of correspondent conjugate was added by a pipette
- After 30 mins of constant stirring, the sample was passed through a 50 kDA membrane filter
- 600 mg of mannitol was dissolved in small amount of distilled water (5 ml) and added to the suspension in order to facilitate spray drying process
- Sample was passed through the spray dryer with temperature set at 96°C
- The final product was stored at room temperature

Loading the NPs with OMVs

Reagents

OMVs	12 mg
Protein P	300 mg
Basic aminoacid	50 mg
Mannitol	600 mg
Ethanol 60%	30 ml
Distilled water	30 ml

For coated NPs :

Compound A/B/AB	750 μl	(previously solubilized)

Procedure

- Protein P and basic aminoacid were dissolved in 30 ml of ethanol 60% under magnetic stirrer
- OMVs were resuspended in 5 ml of H_2O and sonicated at 5 W for 1 min
- 25 ml of distilled water was added to the suspension together with the sonicated antigen. A syringe tube was used to create a costant flow
- In case of preparation of coated NPs 750 μl of a correspondent conjugate was added by a pipette
- After 30 mins of constant stirring, the sample was passed through a concentrator containing a 50 kDA membrane filter
- Mannitol was dissolved in small amount of distilled water (5 ml) and added to the suspension in order to facilitate spray drying process
- Sample was passed through the spray dryer with temperature set at 96°C
- The final product was stored at room temperature

C Detection of OMVs on the surface

Separating the nanoparticles with exposed antigen

Reagents

Dynabeads® Protein G Kit
Specific antibodies against *Shigella flexneri*
PBS
Sample buffer

Samples:	OMV-loaded nanoparticles 100 mg/ml
Control:	Empty nanoparticles 100 mg/ml

Procedure

- One hundred μl of both samples and controls were taken into separate eppendorfs
- Specific antibodies against *Shigella flexneri* were diluted in PBS (1:10), 200 μl of the solution was added to the samples and the mixtures were incubated for 60 min at 37°C
- Dynabeads® Protein G Kit was vortexed properly and 25 μl of the suspension was added to each sample
- After 10 min incubation at room temperature, the samples were placed on a magnet and the supernatant was removed by a pipette
- The separated magnetic particles were used for further examination (SDS-PAGE)

Proteic profile of the separated particles

Reagents

PBS
Sample buffer
Coomassie Brilliant Blue
Revealing solution: 5 % isopropanol, 15 % acetic acid in H_2Od

Procedure

- The separated particles obtained from the previous experiment were resuspended in 40 μl of PBS and 20 μl of Sample buffer
- The samples were vortexed properly and boiled for 5 min
- By boiling in the presence of the Sample buffer, the magnetic beads separated from out particles. By placing a magnet, we could separate the beads again and use the supernatant containing our NPs
- The supernatants were loaded on 12% acrylamide gel and run for 50 min at 200 V
- The gel was washed in Coomassie Brilliant Blue for 2 h and then in Revealing solution for 2 h

Antigenicity of the separated particles

Reagents

Transfer buffer	25 mM Tris base, 192 mM glycine, 10 % methanol
Blocking buffer	5% milk in transfer buffer
Solution A	60 mg of α-chloronaftol, 20 ml of ethanol
Solution B	100 ml of tris-buffered saline (TBS), 60 μl of H_2O_2
Primary antibodies	from rabbit immunized with *Shigella flexneri*
Secondary antibodies	from goat immunized with rabbit IgG, conjugated with peroxidase

Procedure

- Before performing an immunoblotting, the proteins were separated on a acrylamide gel (as described above)
- Instead of staining by Coomassie Blue, the proteins were transferred from the gel to a nitrocellulose membrane:
- The gel and nitrocellulose were placed in between pieces of filter paper soaked in transfer buffer and the proteins were transferred using Trans-Blot® cell at 8 V for 30 min. Afterwards the membrane was soaked in blocking buffer overnight
- Next, the membrane was incubated for 4 h in primary antibodies, diluted 1:100 in the blocking buffer
- After washing by blocking buffer, the membrane was incubated for 1 h with secondary antibodies, diluted 1:1000 in the blocking buffer
- Revealing solution was prepared ex tempore by mixing Solution A and Solution B and the membrane was washed in this revealing solution until clear purple bands occurred

D Fluorescent labelling

Labelling empty NPs with Lumogen®

Reagents

Protein P	300 mg	
Basic Aminoacid	50 mg	
Mannitol	600 mg	
Lumogen® F Red 300	7.2 mg	
Ethanol absolute	18 ml	
Distilled water	42 ml	
Compound AB	750 μl	(previously solubilized)

Procedure

- Lumogen® was dissolved in 18 ml of ethanol absolute under magnetic stirrer and 12 ml of water was added
- Protein P and aminoacid were added and dissolved by stirring
- 30 ml of distilled water and 750 μl of previously solubilized Compound AB were added and the sample was continuously stirred for 30 mins
- Mannitol was dissolved in small amount of water and added to the formulation
- Finally, the sample was passed through the spray dryer with temperature set at 96°C
- The final product was stored at RT

Labelling OMVs with Vybrant® DiO Cell-Labeling Solution

Reagents

OMVs	4 mg
Vybrant® DiO	20 μl
Distilled water	4 ml

Procedure

- Stock suspension of OMVs in water (1 mg/ml) was prepared and sonicated (5 W, 1 min)
- 20 μl of DiO was added, followed by vortex
- The samples was being gently shaked at 37°C for 20 mins and then let recover at RT for 10 mins

E Revealing the encapsulated antigen

Dissolving the proteic cover

Reagents

Ethanol 70%
Ethanol 85 %
DMSO pure

Samples: OMV-loaded nanoparticles 20 mg
Control: Empty nanoparticles 20 mg

Procedure

- First, 20 mg of each nanoparticle type was weighted, resuspended in H_2Od, vortexed and centrifuged (at 10.000 g, 15 min, RT)
- The supernatants were removed, 1.5 ml of ethanol 70% was added to the pellets, the samples were vortexed properly and centrifuged (at 10.000 g, 15 min, RT)
- The supernatants were removed, 1.5 ml of ethanol 85% and 250 μl of DMSO was added to the pellets, the samples were vortexed and centrifuged for the last time (at 10.000 g, 15 min, RT)
- The supernatants were centrifuged and the final pellets were used for the next experiment (proteic and LPS profile)

Proteic profile of the revealed antigen

Reagents

PBS
Sample buffer
Coomassie Brilliant Blue
Revealing solution: 5 % isopropanol, 15 % acetic acid in H_2Od

Procedure

- The pellets obtained from the previous experiment were resuspended in 40 μl of PBS and 20 μl of Sample buffer
- The samples were vortexed properly and boiled for 5 min
- The boiled samples were loaded on 12% acrylamide gel and run for 50 min at 200 V
- The gel was washed in Coomassie Brilliant Blue for 2 h and then in Revealing solution for 2 h

Silver staining

Reagents

Solution A	50% methanol, 40% acetic acid, 40% water
Solution B	7.5% methanol, 5% acetic acid, 92% water
Developing solution	32 g of Developer in 1 l H_2Od
Stopping solution	5% acetic acid in water

Paraperiodic acid

Silver reagent

Procedure

- The pellets obtained from the previous experiment (Dissolving the proteic cover) were resuspended in 40 μl of PBS and 20 μl of Sample buffer
- The samples were vortexed properly and boiled for 5 min
- The boiled samples were loaded on 12% acrylamide gel and run for 50 min at 200 V
- The gel was washed in Solution A for 30 min, then in Solution B for 60 min and then washed with H_2Od for 10 min
- Next, the gel was oxidised for 10 min with a solution of 0.7 % paraperiodic acid in Solution B and washed thoroughly (20 min in H_2Od and 20 min in H_2Odd)
- The silver reagent was then used on the gel for 20 min, followed by washing
- The gel was then soaked in the Developing solution
- After visible brown bands occured, the gel was soaked in the Stopping solution in order to stop the staining reaction

F Instrumental equipment

Copper grid 300 mesh copper grid, EMS, PA, USA

Electron microscope 120 kV ZEISS Libra 120 electron microscope, Zeiss, Germany

Flow cytometer Attune® Acoustic Focusing Cytometer, Life Technologie, CA, USA

Nitrocellulose membrane GE Healthcare life sciences, IL, USA

Rotavapor Büchi rotavapor R-144, Switzerland

Sonicator Microson TM, Misonix, NY, USA

Spectrophotometer Thermo electron corporation, MA, USA

Spray dryer Büchi miniSpray dryer B-290, Büchi Labortechnik, Switzerland

Tangential flow filtration system Pellicon ® XL, Fisher Scientific, PA, USA

Trans-Blot® cell Bio-Rad, CA, USA

ZetaPus Analyzer Brookhaven Instruments Corporation, NY, USA

G Chemicals and reagents

3-(N-morpholino)propanesulfonic acid Bio-Rad, CA, USA

α-chlornaftol Sigma-Aldrich, MO, USA

Acetic acid Pancreac, Spain

Coomassie Blue Bio-Rad, CA, USA

$CuSO_4$ Pancreac, Spain

Developer Bio-Rad, CA, USA

Dimethylsulfoxide pure Pancreac, Spain

Dynabeads® Protein G kit Thermo Fisher Scientific, MA, USA

Ethanol absolute Pharmpur®, Sharlau

Folin-Ciocalteus's reagent Pancreac, Spain

HIO_4 Pancreac, Spain

H_2O_2 Pancreac, Spain

H_2SO_4 Pancreac, Spain

Isopropanol Pancreac, Spain

Lumogen®F Red 300 BASF, Germany

Milk Asturiana Leche en Polvo, Spain

Methanol Pharmpur®, Sharlau

$NaAsO_2$ Pancreac, Spain

Na_2CO_3 Merck, Germany

Para-periodic acid Pancreac, Spain

Phosphate buffer-saline Gibco life technologies, NY, USA

Purified LPS extract from *Shigella flexneri* UNAV, Spain

Sample Buffer Bio-Rad, CA, USA

Silver staining developer Bio-Rad, CA, USA

Silver staining reagent Bio-Rad, CA, USA

Sodium dodecyl sulpahte Sigma-Aldrich, MO, USA

Sodium tartrate Pancreac, Spain

Specific antibodies against *Shigella flexneri* UNAV, Spain

Thiobarbituric acid Pancreac, Spain

Tris-buffered saline Merck, Germany

Tris-Glycine Buffer Bio-Rad, CA, USA

Vybrant® DiO Cell-Labeling Solution Thermo Fisher Scientific, MA, USA